Protein degradation: **The ins and outs of the matter**

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In eukaryotic cells, nascent membrane or secretory proteins are translocated into the endoplasmic reticulum through the Sec61p translocation channel; recent evidence suggests that, if they fail to achieve a native conformation, they are translocated back into the cytosol by the same route and degraded by the proteasome.

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It has long been recognized that misfolded forms of proteins normally destined for secretion or surface expression by eukaryotic cells fail to escape from the endoplasmic reticulum (ER). Misfolding may occur because of a mutation or because the protein is a subunit of a heteromultimeric species and the other subunits are absent. An example of the latter situation occurs in plasma cells, where variants that synthesize immunoglobulin heavy chains, but not light chains, fail to express the heavy chains at the cell surface or to secrete them [1]. Similarly, T-cell receptor α and β subunits are not expressed at the cell surface in the absence of the CD3 components that they normally associate with [2], and major histocompatibility complex (MHC) class I heavy chains are not expressed at the cell surface in the absence of their small subunit partner, β_2-microglobulin [3]. The fate of these misfolded subunits is proteolysis, and for many years researchers have tried to discover the mechanism of ER-associated protein degradation. Recent work has made a strong case that much, if not all, ER-associated proteolysis occurs in the cytosol and is mediated by the major cytosolic protease, the proteasome.

Evidence that cytosolic components are required for ER-associated proteolysis initially came from studies in yeast. In an attempt to replicate the process *in vitro*, McCracken and Brodsky [4] used isolated microsomes containing a post-translationally translocated, non-glycosylated mutant form of pre-pro-α factor, and found that the addition of yeast cytosol and ATP were required to promote the mutant protein's degradation. The implication is that either a cytosolic component is required to mediate or activate proteolysis in the ER lumen, or the misfolded protein substrate has to be made accessible to a protease in the cytosol. Subsequent genetic analysis suggested that the latter interpretation is correct. Werner *et al.* [5] found that cytosol extracted from yeast with mutations in the *pre1* and *pre2* genes, which encode proteasome subunits, can promote protein export from microsomes, but that degradation of the exported protein is substantially reduced. Hiller *et al.* [6], studying the degradation of a mutant form of the soluble vacuolar protein carboxypeptidase yscY, found that, after synthesis, insertion into the ER lumen and glycosylation, the protein was translocated back to the cytoplasm, where the glycosylated form of the enzyme was conjugated with ubiquitin and degraded by the proteasome.

The evidence that similar mechanisms are at work in ER-associated proteolysis in mammalian cells came from a surprising source — studies of the escape mechanisms used by human cytomegalovirus (CMV) to avoid recognition by cytotoxic T cells. Effective recognition of virally-infected cells depends upon the assembly and expression at the cell surface of MHC class I molecules associated with a peptide derived from a viral protein (for reviews, see [7,8]). Assembly occurs in the ER, and involves the MHC-encoded class I transmembrane glycoprotein, the β_2-microglobulin subunit, and a peptide that is generated by cytosolic proteolysis and translocated into the ER by the 'transporter associated with antigen processing' (TAP). Successful assembly satisfies the requirements of ER quality control [9], and the MHC class I–peptide complex is transported from the ER, via the Golgi apparatus, to the cell surface, where cytotoxic T cells can recognize it and kill the virally infected cell.

A number of viruses have evolved ways of disrupting the MHC class I assembly and transport process, and CMV has the most sophisticated set of mechanisms so far discovered. Human CMV encodes a protein, US3, that causes MHC class I molecules to accumulate in the ER and prevents their transport to the cell surface [10,11]. A second CMV protein, US6, interferes with the ability of TAP to translocate peptides from the cytosol to the ER, thus preventing assembly of MHC class I–peptide complexes [12–14]. Two other CMV proteins, US2 and US11, were found by Wiertz, Ploegh and co-workers [15,16] to cause rapid retrotranslocation of MHC class I heavy chains from the ER to the cytosol.

In the presence of proteasome inhibitors, such as lactacystin [17], class I heavy chains were found to accumulate in the cytosol of CMV-infected cells. In contrast to the results in yeast [6], the cytosolic class I molecules were in an unglycosylated form. An *N*-glycanase activity has been described in mammalian cytosol [18], and evidence was obtained suggesting that, in the cytosolic class I heavy

chains, the asparagine residue that serves as anchor for the glycan moiety was converted to an aspartic acid, consistent with deglycosylation by such an enzyme. This led to the hypothesis that MHC class I heavy chains enter the ER lumen, acquire their *N*-linked glycan and, in some manner mediated by US2 or US11, are shunted back into the cytosol where they are deglycosylated and rapidly degraded by the proteasome. Only in the presence of proteasome inhibitors are the deglycosylated chains detectable.

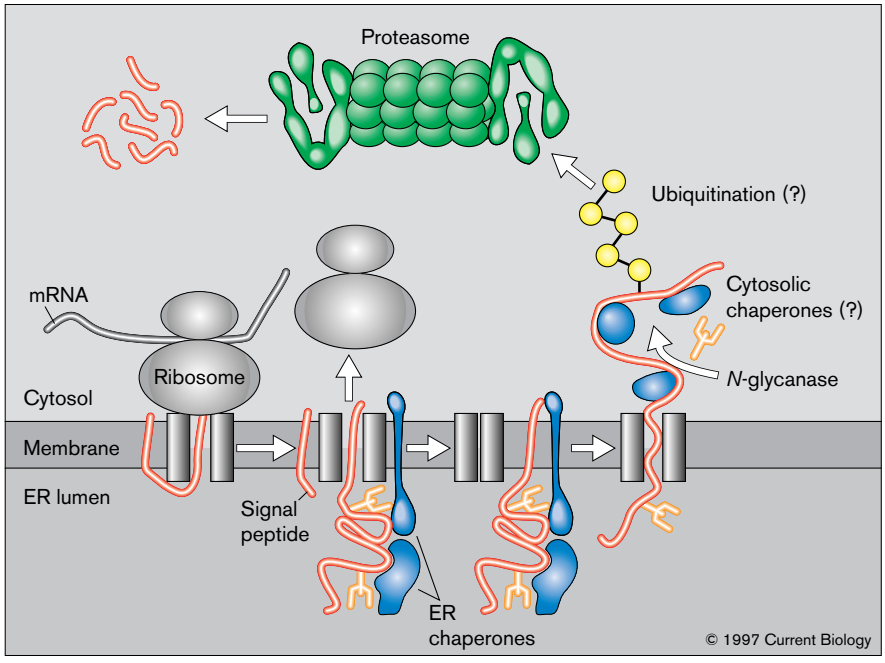
In cells making US2 or US11, the rate of retrotranslocation of MHC class I heavy chains is extremely rapid — deglycosylated heavy chains are detectable in the cytosol within minutes of their synthesis [15,16]. The degradation of MHC class I molecules that occurs in the absence of β_2-microglobulin, or of peptides supplied by TAP, is much slower, raising the question of whether this degradation occurs in a similar manner to that induced by the CMV genes. Subsequent experiments showed that indeed it does. Mutant human cell lines lacking either TAP or β_2-microglobulin expression were found also to accumulate deglycosylated MHC class I heavy chains in the cytosol in the presence of proteasome inhibitors [19]. The rate of cytosolic accumulation in these cases was again much slower than in *US2* or *US11* expressing cells. A small, but variable, fraction of class I heavy chains in normal cell lines behaved similarly [19], suggesting that peptides might be limiting for class I assembly. Furthermore, when proper folding of class I molecules was impeded by altering the redox potential of the ER with the reducing agent dithiothreitol, they were similarly translocated into the cytosol [15]. Interaction of the translocated proteins with cytosolic chaperones, such as heat shock protein 70 (Hsp70), may be required to maintain their solubility in the aqueous cytosolic environment.

From the above experiments, it is now reasonably well established that the retrograde translocation of misfolded proteins from the ER to the cytosol, and their subsequent proteolysis by the proteasome, accounts for much of what is considered ER-associated protein degradation (Figure 1). This leads, of course, to additional important questions, the first of which is: what is the route by which misfolded proteins are expelled from the ER? The major route of access for proteins into the ER lumen from the cytosol is the Sec 61 complex, the translocation channel responsible for the signal-sequence-dependent entry into the ER of proteins synthesized by membrane-associated ribosomes (reviewed in [20]). The extreme rapidity of US2/US11-induced retrotranslocation of MHC class I heavy chains into the cytosol led Wiertz *et al.* [15] to suggest that the reverse route might also involve the translocation channel.

Integral membrane proteins with amino-terminal signal sequences are introduced into the translocation channel during translation by membrane-associated ribosomes. The signal sequence is cleaved in the ER and *N*-linked glycans are co-translationally added to appropriate asparagine residues. When translation stops, the ribosome dissociates and the protein is thought to move laterally out of the channel by an unknown gating mechanism, allowing the hydrophobic transmembrane domain to integrate

Figure 1

The retrotranslocation of a misfolded membrane glycoprotein from the ER to the cytosol and its degradation by the proteasome. After signal sequence cleavage, the completed glycoprotein moves out of the translocation channel into the ER membrane. Interaction with ER chaperones normally facilitates folding. In this case, proper folding fails, and the molecule reinserts into the translocation channel and is translocated back into the cytosol, where it is deglycosylated and degraded by the proteasome.



into the ER membrane. Wiertz *et al.* [15,16] suggested that the US2 and US11 proteins bind to the newly translocated chains prior to their movement out of the channel, and induce them to slide out into the cytosol.

There is nothing about this suggestion that contradicts current ideas about how the directionality of translocation into the ER is maintained. The directionality of translocation may normally be maintained simply by a combination of ongoing translation and the sequential binding and release of ER chaperones, such as BiP. The binding and release of chaperones are thought to prevent the accidental sliding of proteins back into the cytosol, so that the nascent protein is translocated inexorably in one direction by a kind of Brownian ratchet effect [21]. Theoretically, the US2 and US11 proteins could cause retrotranslocation by preventing chaperone binding, while being insufficiently bulky to prevent a US2–class I or US11–class I complex from falling into the cytosol. Alternatively, a more active process could be involved.

Wiertz *et al.* [15] obtained evidence that the translocation channel is indeed the mediator of retrotranslocation in the CMV system. Both US2 protein and class I heavy chains were found to be physically associated with the translocation channel when proteasome inhibitors were added. In each case, only deglycosylated forms were bound, arguing that both US2 and class I molecules were accessible to the *N*-glycanase enzyme prior to their release into the cytosol. Deglycosylated US2 protein and class I heavy chains were also found associated with the proteasome. This led to the suggestion that US2 binds to newly synthesized MHC class I molecules, perhaps co-translationally, and that the two remain associated during retrotranslocation, deglycosylation and targeting to the proteasome for degradation.

Does retrotranslocation of misfolded proteins from the ER to the cytosol normally involve the translocation channel? The rate of degradation of misfolded class I heavy chains is relatively slow in the absence of US2 and US11. In TAP-negative cells, MHC class I molecules form dimers with β_2-microglobulin prior to degradation [19], and US2 can bind to some β_2-microglobulin-associated class I heavy chains, which presumably are later degraded [15]. These partially assembled class I molecules have almost certainly left the translocation channel and integrated into the ER membrane prior to their degradation. Thus, if the channel is the normal route for retrotranslocation, a mechanism must exist for reinserting completed molecules back into the channel. Powerful genetic evidence that the translocation channel is involved in normal ER-associated degradation has now been obtained [22].

The new work, by Romisch and co-workers [22], uses temperature-sensitive yeast *sec61* mutants. The translocation channel is a complex multimer, consisting of the polytopic membrane protein Sec61p and two additional subunits [20,23]. Mutants defective in Sec61p cannot import proteins into the ER. Using microsomes from *sec61* temperature-sensitive strains, mutant proteins could be imported into the ER lumen at the permissive temperature, and their export and degradation analyzed using the cell-free assay developed by McCracken and Brodsky [4].

The export of mutant pre-pro-α factor from the ER to the cytosol was inhibited in the *sec61* mutants even at a temperature permissive for import. It was also shown that mutant, but not wild-type, pre-pro-α factor could be chemically cross-linked to Sec61p and that this binding was enhanced in the *sec61* mutants. This suggests that misfolded secretory proteins associate with the translocation channel prior to retrotranslocation, whereas normal proteins permanently lose the interaction once translocation and folding are completed.

What mechanisms govern the re-entry of misfolded proteins into the translocation channel? It seems likely that the system of ER chaperones responsible for ER quality control [9] will prove to be intimately involved. Suggestively, the initial work of McCracken and Brodsky [4] showed that microsomes from yeast lacking the ER chaperone calnexin were impaired in their ability to degrade mutant pre-pro-α factor in the presence of cytosol and ATP. Additional components that can modify the Sec61p translocation channel so that it becomes a retrotranslocation channel are likely to be discovered in the future.

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